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Concentrating Capacity of the Human Reduced Folate Carrier (hRFC1) in Human ZR-75 Breast Cancer Cell Lines

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ABSTRACT. Human RFC1 (hRFC1) transfected in transport-deficient methotrexate MTX^RZR-75-1 human breast carcinoma cells (MTX^RZR-75/RFC) were used to investigate the impact of hRFC1 overexpression on influx and concentrative transport of methotrexate (MTX). Eight-fold overexpression of hRFC1, as determined by northern analysis, resulted in a 4-fold increase in MTX influx accompanied by a 2.4-fold increase in the steady-state level of free drug as compared with wild-type ZR-75-1 cells when the extracellular MTX level was 0.5 μM. When extracellular MTX was increased to 10 μM, the increase in influx equaled the increase in the transmembrane chemical gradient for MTX in the transfectant relative to wild-type cells. By 50 min, ~16–20 and 25% of the intracellular ^3H represented MTX polyglutamates by HPLC analysis at $[MTX]_e$ = 0.5 and 10 μM in wild-type and transfected cells, respectively. Overexpression of hRFC1 enhanced sensitivity to MTX in MTX^RZR-75-1 cells by more than 250-fold. The data indicate that overexpression of hRFC1 in human cells results in comparable increases in influx and transmembrane gradients. This is different from what was reported when mouse RFC1 was transfected into murine leukemia cells, resulting in large, more symmetrical increases in the MTX bidirectional transport kinetics with a much smaller change in steady-state levels. The changes in the human cells transfected with hRFC1 however, were similar to what has been observed by other investigators when RFC1 expression is increased by low folate selective pressure. BIOCHEM PHARMACOL **55**;10:1683–1689, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. transport; methotrexate; reduced folate carrier; folate

The reduced folate carrier is a major route of transport for natural folates such as 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, and a variety of folate analogs [1–4]. The carrier, recently cloned as RFC1§ [5–10], achieves uphill transport of folates into mammalian cells through an anion exchange mechanism [2, 11–15]. There are several other potential routes of transport of folates into cells of lymphoid origin. These include GPI-anchored membrane folate binding proteins that mediate entry via an endocytotic mechanism [4, 16–20], and a transport process that becomes active at low pH [21, 22]. Of particular importance is the exit pump(s), directly coupled to energy metabolism, that drives folates out of the cell, opposing concentrative transport mediated by RFC1 at physiological pH [23–25].

In a recent study from this laboratory, the impact of high carrier expression, achieved by transfection of murine RFC1, on the concentrative transport of MTX was assessed in L1210 leukemia cells in which endogenous carrier was not functional [26]. Increased carrier expression resulted in large increases in the bidirectional transport kinetics of MTX with a much smaller change in the steady-state transmembrane gradient achieved. This finding was considerably different from what was observed when carrier expression was increased by low folate selective pressure [27–29]. Under the latter conditions, there was a large increase in both MTX influx and the transmembrane gradient without any change in efflux kinetics at all. In this paper, we extend this analysis of RFC1 function to evaluate the consequences of transfection of hRFC1 on MTX influx and concentrative transport in a human breast cancer cell line that lacks endogenous carrier.

MATERIALS AND METHODS

Cell Lines and Growth Conditions

ZR-75-1 breast carcinoma cells [30], the MTX-resistant MTX^RZR-75-1 subline [31], and the hRFC1 transfectant MTX^RZR-75/RFC [6] were maintained in 25 cm^2 culture

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§ *Abbreviations:* RFC1, reduced folate carrier; hRFC1, human RFC1; GPI, glycosylphosphoinositol; MTX, methotrexate; IMEM, improved Minimum Essential Medium (Richter's modified); IMDM, Iscove's modified Dulbecco's medium; DHFR, dihydrofolate reductase; and $NADP^+$, oxidized nicotinamide-adenine dinucleotide phosphate.

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flasks in IMEM or IMDM supplemented with 5% fetal bovine serum, 2 mM of L-glutamine, 100 units/mL of penicillin and 100 μg/mL of streptomycin. Hygromycin B (150 μg/mL) was added to the medium of the transfected cells.

Analysis of MTX Transport in Monolayer Cultures

Cells were grown to mid-log phase at the base of 17-mm glass scintillation vials (Research Products International) containing 1.0 mL of medium. Uptake studies were carried out in a 37° water bath. Transport measurements were initiated after aspiration of growth medium from the vial and addition of transport medium (IMDM, 20 mM of HEPES, pH 7.4) containing [^{3}H]-MTX. Uptake was stopped by immersing the vials in ice-water, aspiration of the transport medium, followed by the careful addition of, and washes with, ice-cold PBS (pH 7.4). For measurements of bound intracellular MTX, cells were loaded with [^{3}H]MTX for 10 min, the medium was replaced with MTX-free transport buffer, and efflux was continued for up to 40 min before addition of, and washes with, ice-cold PBS. Cells were further processed by the addition of 0.5 mL of 0.2 N NaOH to the vial and incubation at 65° for 30 min. A portion of the lysate was used to determine protein by the BCA Protein Assay Reagent kit (Pierce), and 400 μL of the cell lysate was assayed for ^{3}H by liquid scintillation spectrometry.

Free intracellular drug was calculated as follows: The level of polyglutamates at any time was determined using the linear regression equation $y = mx + b$, where y is the intracellular MTX-polyglutamate level, b is the zero time intercept, and m is the uptake slope during the linear portion beyond 30 min. In this study, y is set to zero at 8 or 3 min for $[MTX]_e = 0.5$ or 10 μM, respectively (based on the observation that polyglutamylation is initiated shortly after DHFR saturation). The level of polyglutamates so determined was subtracted from total intracellular MTX for each uptake point to obtain the monoglutamate component. Following correction for MTX bound to DHFR, as assessed by drug efflux prior to accumulation of polyglutamates, the steady-state level of free MTX was established.

Determination of Intracellular Water

The cell suspension was incubated with [*carboxyl*-^{14}C]inulin in transport medium for 10 min at room temperature. The cell sample was divided and centrifuged for 10 min, and the supernatant was carefully removed. The extracellular inulin concentration was determined on a portion of the supernatant (see below). The wet cell pellets were weighed and dried overnight at 65°, and dry weights were obtained. Then the dry pellets were solubilized in 0.4 mL of 1 M of KOH at 65° for 30 min, fluor was added, and radioactivity was determined in the pellet hydrolysate as well as the original transport medium. Extracellular water of the pellet was computed from the ratio of inulin associated with the cell pellet to the buffer inulin concentration. Intracellular water was the difference between the wet and dry weights of the pellet less the extracellular water. For measurement of the protein to mass ratio, the cell pellet was dried overnight at 65°, weighed, and solubilized in 1 M KOH at 65° for 1 hr. Total protein was determined as indicated above, and intracellular water per milligram of protein was computed. The concentration of drug in the intracellular water was determined from the separate measurement of the level of MTX per milligram of protein.

Analysis of MTX Polyglutamates

Monolayer cultures of wild-type or MTX^{R}ZR-75/RFC cells were grown to mid-log phase in 6-cm tissue culture plates. Uptake of MTX was initiated by replacing the growth medium with [^{3}H]MTX-containing transport medium followed by incubation at 37° for 50 min. Uptake was terminated by removal of the medium and replacement with ice-cold PBS. The plates were washed three additional times in ice-cold PBS, following which cells were scraped off the plate with a plastic policeman and thoroughly resuspended in 1 mL of PBS. A portion (0.8 mL) of the cell suspension was extracted with 20% trichloroacetic acid for 10 min at 0° and centrifuged for 10 min at 4°. The supernatant thus recovered was neutralized by addition of 0.5 mL of 1 M of KH_2PO_4 and 0.3 mL of 6 M of NaOH and analyzed for polyglutamate derivatives of MTX [32] by HPLC using two linear gradients of 0–10 and 10–15% acetonitrile over 35 and 15 min, respectively. Authentic MTX-$Glu_{(1-6)}$ standards (Schircks Laboratories) were included and monitored by UV absorbance at 280 nm. MTX-$Glu_{(6-3)}$ eluted in 0–10% acetonitrile, and MTX-$Glu_{(2-1)}$ resolved in 10–15% acetonitrile.

DHFR Assay

DHFR was assayed spectrophotometrically, utilizing the decrease in absorbance at 340 nm as NADPH is oxidized to $NADP^+$ [33]. Total protein in the cell lysate was determined as described above. Units of enzyme activity were calculated as described by Mathews *et al.* [34].

Assessment of Growth Inhibition

Cytotoxic effects of MTX on ZR-75-1, MTX^{R}ZR-75-1, and hRFC1 transfected MTX^{R}ZR-75/RFC cells were determined by sulforhodamine B staining with slight modification of the procedure of Skehan *et al.* [35].

Northern Analysis

Total RNA from ZR-75-1, MTX^{R}ZR-75-1, and MTX^{R}ZR-75/RFC cells was isolated using the RNase midi kit (QIAGEN). RNA (10 μg) was fractionated on a 1% denaturing formaldehyde-agarose gel, transferred to Nytran membrane (Schleicher & Schuell), and immobilized by UV

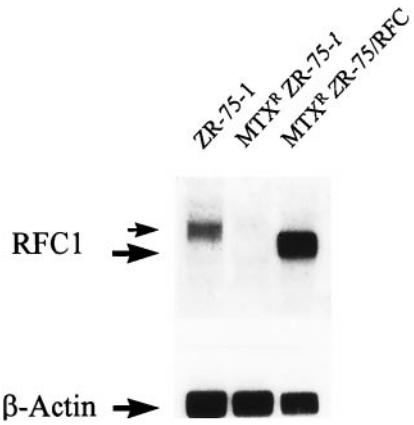

FIG. 1. Representative Northern blot analysis of total RNA isolated from human breast cancer cell lines: ZR-75-1, MTX^RZR-75-1, and MTX^RZR-75/RFC. The RNA blot was probed with the full-length hRFC1 cDNA and subsequently with mouse β-actin cDNA. The endogenous RFC1 transcript and the one produced from the expression vector are indicated by small and large arrows, respectively. RFC1 transcripts were quantitated by PhosphorImager analysis and normalized with the β-actin RNA levels.

cross-linking (Stratalinker UV cross-linker, Stratagene). The blot was probed with the full-length hRFC cDNA and reprobed with mouse β-actin cDNA. Hybridization and washing were performed as previously described [36].

RESULTS

Effect of Increased Expression of hRFC1 on MTX Influx, Net Transport, and Formation of Polyglutamyl Derivatives

hRFC1 mRNA expression was assessed on total RNA isolated from ZR-75-1, MTX^RZR-75-1, and MTX^RZR-75/RFC cells by northern analysis. The transfected mRNA contains only the coding region of the hRFC1 cDNA, has a lower molecular weight, and hence migrates faster, than the endogenous hRFC1 mRNA. There was an 8-fold increase in RFC1 mRNA in the transfected MTX^RZR-75/RFC as compared with the wild-type line (Fig. 1). The resistant line, MTX^RZR-75-1, showed no detectable RFC1 message.

MTX influx in MTX^RZR-75/RFC cells was 4-fold greater than in the ZR-75-1 line when the extracellular MTX level was 0.5 μM (Fig. 2A, insert). Increasing the extracellular MTX level 20-fold to 10 μM (Fig. 2B, insert) increased influx only 4-fold in both ZR-75-1 and MTX^RZR-75/RFC cells, consistent with a saturable influx process [23]. Net transport of MTX was also increased in MTX^RZR-75/RFC cells as compared with the ZR-75-1 line when the extracellular MTX level was 0.5 and 10 μM. Net uptake, however, did not reach steady state over the interval studied; rather, uptake fell to a slower, nearly constant velocity over the interval of 30–60 min after addition of MTX. Previous studies established that this late uptake component represents the rate of formation of polyglutamyl

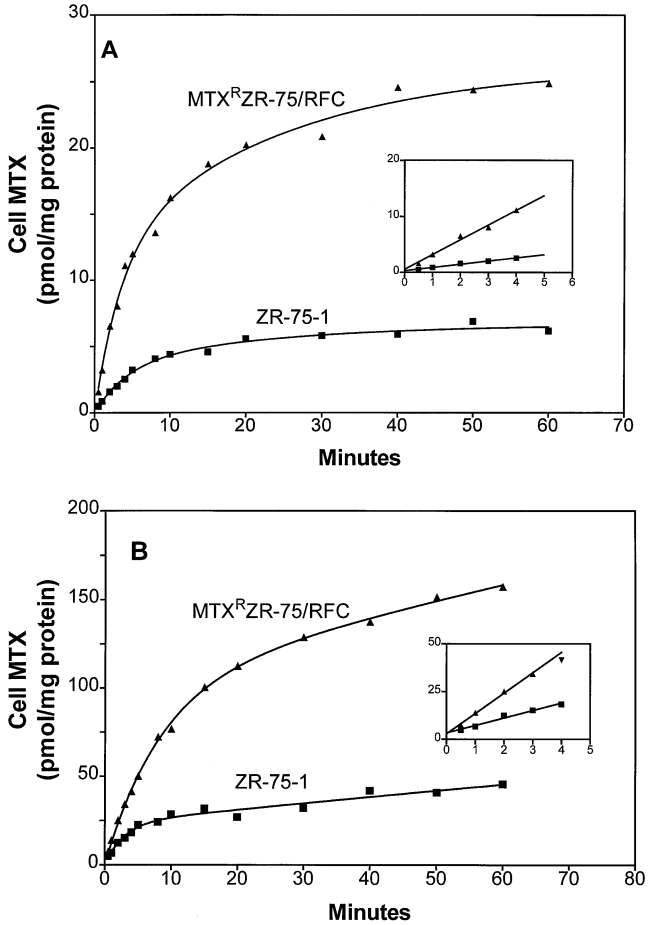

FIG. 2. Representative time course of MTX uptake in ZR-75-1 and MTX^RZR-75/RFC cells. Cells were incubated with [^{3}H]MTX at time zero. Initial rates are shown in the inset. Panel A: 0.5 μM of [^{3}H-MTX]$_e$; Panel B: 10 μM [^{3}H-MTX]$_e$.

derivatives retained within the cells, which achieves a constant velocity when the free intracellular monoglutamyl level is at steady state [32, 37].

The extent of formation of MTX polyglutamate derivatives was confirmed by HPLC analysis of intracellular ^{3}H after incubation of cells with [^{3}H]MTX for 50 min (Fig. 3). At $[MTX]_e$ = 0.5 μM, 6.96 (24%) and 1.79 pmol of MTX/mg of protein (20%) out of a total antifolate level of 29.0 and 8.9 pmol MTX/mg protein represented polyglutamate derivatives in MTX^RZR-75/RFC and wild-type ZR-75-1 cells, respectively. When extracellular MTX was 10 μM, 39.6 (27%) and 6.56 pmol MTX/mg of protein (16%) out of a total of 145.6 and 41 pmol MTX/mg of protein represented polyglutamyl derivatives in the transfected and wild-type ZR-75-1 cells, respectively.

When the data in panels A and B of Fig. 2 were corrected as described in Materials and Methods, based upon the uptake slope over 30–60 min, for the contribution of MTX polyglutamates to the total level of MTX in the cells at each uptake point, free monoglutamate achieved steady state in the intracellular water by 10–15 min (Fig. 4, A and B). Free intracellular MTX was then computed by subtracting the component bound to DHFR (1.87 and 10.63 for the

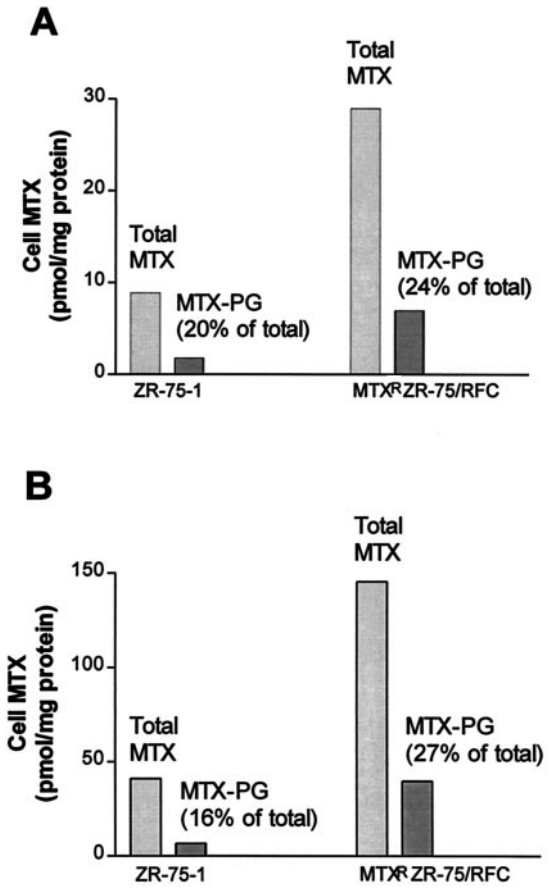

FIG. 3. HPLC analysis of MTX polyglutamyl derivatives in ZR-75-1 and MTX^{R}ZR-75/RFC cells. Cells were incubated with [^{3}H]MTX for 50 min under usual growth conditions. The lysates were analyzed for mono and polyglutamyl derivatives as described in Materials and Methods. Data are the averages of three experiments. Panel A: 0.5 μM of [^{3}H-MTX]$_e$. Panel B: 10 μM of [^{3}H-MTX]$_e$.

wild-type and the transfectants, respectively), as determined by the level of retained intracellular MTX when cells loaded with drug are incubated in MTX-free medium before polyglutamates have accumulated. The observed difference in DHFR levels in the two lines was also confirmed by enzymatic measurement: 9.69 and 73.9 units of DHFR/mg protein in the wild-type and transfectants, respectively.

Results of an analysis of the various cell MTX components and the free intracellular concentrations are shown in Table 1. When extracellular MTX was 0.5 μM, steady-state intracellular MTX was 0.56 μM in wild-type cells and 1.37 μM in the transfectant—a 2.4-fold difference. This increase in free intracellular MTX was only 60% of the 4-fold increase in influx in the MTX^{R}ZR-75/RFC cells. When extracellular MTX was 10 μM, the steady-state free intracellular MTX concentration in wild-type cells was 3 μM, increasing 3.5-fold to 10.6 μM in the transfected cells, a level comparable to the increase in influx.

Impact of hRFC1 Expression on MTX Inhibition of Cell Growth

Transfection of hRFC1 to produce the MTX^{R}ZR-75/RFC line rendered these cells 250-fold more sensitive to MTX than MTX^{R}ZR-75-1 cells but still an order of magnitude resistant to drug in comparison with wild-type ZR-75-1 cells (Fig. 5). This residual resistance was within the same order as the 8-fold higher DHFR enzyme activity and the 6-fold increase in the MTX binding capacity of these cells.

DISCUSSION

In murine leukemia cells, the reduced folate carrier produces only small transmembrane chemical gradients, but when the membrane potential is considered, very large electrochemical-potential differences for folates, as characterized best for MTX, are generated [1, 23]. The energy for this uphill transport derives from the transmembrane organic anion gradient through an exchange mechanism in which the downhill flow of anions out of the cell is coupled to the uphill flow of MTX into cells via the carrier [11–15]. If this were the only route for MTX transport under conditions in which the organic anion gradient and membrane potential are constant, the transmembrane MTX gradient should be independent of the number of transport carriers within the cell membrane. Hence, increased carrier expression would increase the rate of transport into and out of the cells, and the rate at which the system reaches steady state, but the final gradient achieved would not change. Alterations in the transmembrane folate gradient could only occur if there was a parallel transport route through which folates can exit the cell. Hence, the extent of change in the transmembrane gradient that occurs with changes in carrier expression would be dependent upon the relative magnitude of the carrier-mediated and parallel fluxes. In virtually all mammalian cells studied, there is at least one exit route that mediates efflux of MTX in addition to the carrier. This is an ATP-dependent process that is blocked by metabolic poisons and a variety of agents that presumably utilize this pathway [23–25]. The results of the studies reported in this paper will be interpreted within the context of this paradigm.

Transfection of hRFC1 into transport-deficient human MTX^{R}ZR-75-1 breast cancer cells resulted in an 8-fold increase in message but only a 4-fold increase in MTX influx. While the level of transport protein could not be assessed, this is not unlike the discrepancy between the high-level expression of both message and membrane carrier binding capacity and the much lower increase in influx in K562 hRFC transfectants [38]. The change in influx was accompanied by a 2.4-fold increase in the steady-state intracellular MTX level when the extracellular MTX was 0.5 μM. Increasing extracellular MTX to 10 μM resulted in a 3.5-fold increase in influx and the transmembrane gradient in the transfectant. Because the change in influx is quantitatively similar to the increase in the steady-state

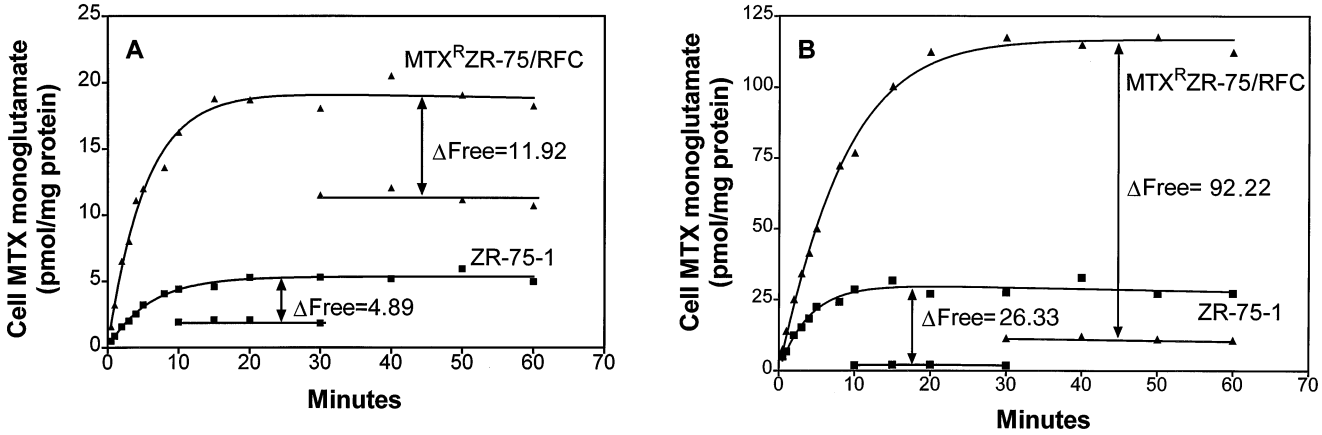

FIG. 4. Uptake of MTX corrected for polyglutamylation. The fraction of MTX polyglutamates was determined and subtracted from total antifolate at each uptake point as detailed in Materials and Methods. Bound levels of MTX were determined from the level of MTX remaining in cells upon resuspension into drug-free medium prior to the formation of polyglutamate derivatives. The arrows indicate the exchangeable (or free) intracellular MTX levels. Panel A: 0.5 μM of $[^3H\text{-MTX}]_e$, Panel B: 10 μM of $[^3H\text{-MTX}]_e$.

MTX level, particularly at high extracellular MTX concentrations, this must be due to an asymmetrical change in the bidirectional transport properties; influx kinetics are increased with little net change in efflux kinetics mediated by the carrier and exit pump(s). The nature of the monolayer system studied, however, precluded quantitation of efflux properties of these cells. MTX sensitivity increased 250-fold in the transfected cells, but they remained 10-fold resistant to drug compared with the wild-type ZR-75-1 cells. This can be attributed to the high level of DHFR observed in the transfectant cell line. Earlier studies [31, 39] reported that the enzyme level in the MTX^RZR-75 cells, in which the hRFC1 was transfected, is the same as in the wild-type cells. The increase in the enzyme activity observed here might, therefore, be a result of the transfection and selection conditions.

These changes for hRFC1 in human cells are quantitatively different from what was observed with mouse RFC1 transfected into murine leukemia cells [26]. Here the increase in RFC1-mediated influx was higher; however, the changes observed were more symmetrical. Hence, at low extracellular MTX levels, the influx V_{max} was increased by a factor of nine, the efflux rate constant was increased by a factor of five, and the steady-state gradient was increased by only a factor of two. While in that study the impact of carrier expression on transmembrane gradients achieved increased as the extracellular MTX concentration increased, the change in steady-state level was always far less than the influx change.

The data indicate (Table 1) that the transmembrane gradients for MTX decrease as the extracellular drug concentration is increased in parental and transfected breast cancer cell lines. This is well established for MTX transport in L1210 cells [1, 23, 26]; intracellular drug approaches a maximum level, consistent with an absorption isotherm, resulting in a fall in the steady-state transmembrane gradient with increasing extracellular drug levels. This is characteristic of uphill carrier systems in which there is an asymmetry in the affinity of carrier for its substrates, or differences in the concentrations of competing substrates, across the cell membrane. In the case of the murine RFC1, the K_m for efflux is so much higher than for influx that

TABLE 1. Analysis of MTX influx and steady-state levels within human breast cancer cells

	$[MTX]_e$ = 0.5 μM					$[MTX]_e$ = 10 μM				
	ZR-75-1	N	MTX^RZR-75/RFC	N	Fold difference	ZR-75-1	N	MTX^RZR-75/RFC	N	Fold difference
Total MTX (pmol/mg protein)	6.76 ± 0.51	5	22.55 ± 1.01	5	3.33	28.20 ± 0.20	3	102.85 ± 9.00	3	3.65
Bound MTX (pmol/mg protein)	1.87 ± 0.087	8	10.63 ± 0.37	7	5.68	1.87*		10.63		
Free MTX (pmol/mg protein)	4.89 ± 0.51	5	11.92 ± 1.01	5	2.44	26.33 ± 0.30	3	92.22 ± 9.00	3	3.5
$[MTX]_i$ (μM)	0.56		1.37		2.44	3.03		10.62		3.5
$[MTX]_i/[MTX]_e$	1.12		2.74		2.44	0.30		1.06		3.5
Influx (μmol/L/min)	0.66 ± 0.06	6	2.83 ± 0.22	6	4.1	3.78 ± 0.64	3	13.47 ± 1.60	3	3.56

Transport parameters were measured at $[MTX]_e$ = 0.5 and 10 μM, as indicated. Free intracellular MTX levels were determined by correcting for the level of polyglutamate derivatives and drug bound to DHFR as described in Materials and Methods. Data are the means ± SEM of (N) experiments performed on different days.

*Considered to be the same as measured at $[MTX]_e$ = 0.5 μM.

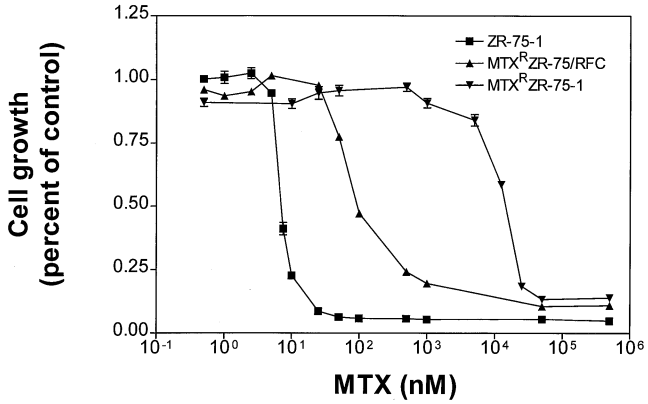

FIG. 5. Cytotoxicity of MTX to ZR-75-1, MTX^{R}ZR-75-1, and MTX^{R}ZR-75/RFC cells. Cells (1000) were grown in 96-well plates with increasing amounts of MTX for 6 days, and growth was measured by sulforhodamine B staining as described in Materials and Methods. Data are the means ± SEM of 4 different wells in a representative experiment.

efflux is essentially first order [1]. Further, the greater augmentation of transmembrane gradients for MTX achieved in the transfectants, as the extracellular substrate level is increased in the breast cancer lines, can be attributed to increased saturation of the carrier and increased transport by RFC1 relative to the exit pump(s) at high extracellular drug levels. While this relationship is observed also for mRFC1 transfected in L1210 leukemia cells [26], as discussed above, the maximum increase in the steady-state gradient observed and predicted remains far less than the change in influx.

The basis for this difference between the hRFC1 and mRFC1 is not clear. There are differences in the predicted RFC1 proteins between the human and mouse carriers that may account for the different transport properties. While the two proteins have 65% homology, there are an additional 76 C-terminal amino acids present in hRFC1 [5–7, 9, 10], and this, along with other structural differences, may account for the differences observed. On the other hand, there may be unique properties of the host cell membranes that account for the results. Expression of hRFC1 in hamster versus human cells resulted in differences in influx kinetics that reflect properties of both species [7]. It is, therefore, possible that hRFC1 expressed in mouse L1210 cells may exhibit different concentrative properties than hRFC1 expressed in human cells. On the other hand, it is expected that differences in the magnitude of the carrier-mediated fluxes relative to exporter activity would account for differences in the degree of concentrative transport achieved with increased carrier expression. For instance, the higher the contribution of the export pump to the overall efflux of MTX, the greater the increase in steady-state drug levels with enhancement of the carrier-mediated component. This relationship may be more characteristic of human than murine cells. Finally, there may be secondary alterations in the exit pump that modulate changes in the steady-state MTX levels achieved—effects that may be species- and/or cell-specific.

Of interest are studies in which endogenous reduced folate carrier was overexpressed under conditions of low leucovorin selective pressure in human and murine lymphoid and erythroleukemia cells [27–29]. In these cases, marked increases in influx were associated with comparable increases in steady-state levels, with no change in efflux kinetics. This is a pattern more consistent with what is seen in the human breast cancer line transfected with hRFC1 than in mouse lines transfected with mRFC1.

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